Incubation of Bovine Thyroid Slices with Thyrotropin is Associated with a Decrease in the Ability of Pertussis Toxin to Adenosine Diphosphate-Ribosylate Guanine Nucleotide Regulatory Component(s)

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Pretreatment of bovine thyroid slices with TSH resulted in desensitization of TSH-sensitive adenylyl cyclase activity but no change in stimulatory nucleotide binding regulatory component of adenylyl cyclase (G_s) activity assessed by reconstitution of the G_s-defective *cyc*$^-$ S49 adenylyl cyclase system. Possible changes in substrates for pertussis toxin (PT)-induced ADP ribosylation due to TSH treatment and/or in endogenous ADP ribosylation of membrane proteins were explored. Using 10 μM [^{32}P]NAD$^+$ as substrate, endogenous ADP ribosylation was not observed in membranes from control or TSH-treated slices. ADP ribosylation of α-subunits of G_s by cholera toxin was also unaffected by incubation of thyroid slices with TSH. In contrast, ADP ribosylation of 40 kilodalton (kDa) substrates for PT was decreased between 40% and 60% by TSH treatment. This effect of TSH was dependent on its concentration and the time of incubation of the slices and was specific for labeling of the 40 kDa PT substrate. Prostaglandin E_1 treatment of thyroid slices, which results in a much smaller homologous desensitizing effect, did not result in changes in ADP ribosylation by PT. The effect of incubation of slices with TSH was abolished by pretreatment of the membranes with 0.3–1.0% Lubrol PX, which increased the labeling of the 40 kDa polypeptides. The data suggests that TSH induces in thyroid tissue a redistribution of 40 kDa polypeptides changing their availability to PT. (Molecular Endocrinology 1: 482–490, 1987)

INTRODUCTION

An initial incubation of thyroid slices with TSH is associated with a reduction in the response of the adenylyl cyclase system and several other metabolic parameters to the subsequent addition of the hormone (1–4). The adenylyl cyclase-transducing system contains receptors, a dually regulated catalytic unit, C, and at least two guanine nucleotide-binding regulatory proteins (G): stimulatory G (G_s) and inhibitory (G_i) (5–7). G_s transduces hormone occupancy of stimulatory receptors into a GTP-dependent activation of C resulting in increased cAMP formation (8). Receptor promoted inhibition of C activity is transduced by G_i, also in a GTP-dependent manner (9, 10). G_i and G_s are ADP ribosylated by pertussis toxin (PT) and cholera toxin (CT), respectively (11, 12).

In the thyroid, TSH stimulates adenylyl cyclase activity via a G_s signal-transducing protein (13), and increased cAMP levels are thought to mediate several effects of this hormone (14). Inhibitory regulation of adenylyl cyclase has also been shown in the thyroid (15–17), and the presence of both G_s and G_i-type polypeptides in thyroid membranes has been documented (18).

The mechanism(s) of TSH desensitization of adenylyl cyclase has not been completely elucidated. Although there have been reports of decreased binding of TSH in refractory thyroid tissue (19), this was not found in other studies (3, 20). Since forskolin stimulation of the enzyme was unaffected by desensitization, the catalytic activity appeared to be intact. Hence, an altered coupling of the receptor to the adenylyl cyclase system has been postulated (2, 3). In addition, TSH has been reported to enhance endogenous ADP ribosylation of acceptor proteins in isolated thyroid cells and membranes (21, 22). Although ADP ribosylation of G proteins has been implicated in TSH desensitization (23), this was not confirmed (24). TSH treatment of thyroid slices or cells did not cause a change in the *cyc*$^-$-reconstituting ability (2) of such treated membranes or in their levels of ADP-ribosylatable CT substrate (3). Such studies tend to exclude change in G_s in the mechanism of

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TSH-induced desensitization, but it is possible that alterations in G_i might be important. While these studies were in progress, Hirayu *et al.* (25) reported that PT did not modify such TSH-mediated refractoriness (25). We have recently identified in thyroid membranes a protein of approximately 40 kilodaltons (kDa) that serves as substrate for PT catalyzed ADP ribosylation (18). The function of the thyroid PT substrate is not known but we believe it to be a G_i-type protein, possibly involved in inhibitory regulation of adenylyl cyclase. Studies by Rich *et al.* (26) with MDCK cells in culture, showed that desensitization of the adenylyl cyclase system by glucagon increased the susceptibility of PT substrate(s) to ADP ribosylation by the toxin (26).

In contrast to the finding of Rich *et al.* (26), we report a decrease in the ability of PT to ADP ribosylate the thyroid membrane 40 kDa substrate(s) after incubation with TSH. Interestingly, this decrease is due to a masking of the substrate that is relieved by disruption of the membrane structure with the detergent Lubrol PX.

RESULTS

Effects of TSH

The data in Fig. 1A confirm previous findings (1) that an initial incubation of thyroid tissue with TSH diminishes the subsequent stimulation of adenylyl cyclase primarily by TSH, and to a lesser extent also by NaF (3). The decrease is not due to an alteration in the activity of G_s, extracts of control and desensitized membrane reconstituted *cyc*$^-$ adenylyl cyclase activity to the same extent (Fig. 1B), confirming previous results (2). Further, the efficiency of the extraction procedure was similar for both control and desensitized membranes (Fig. 1B). Labeling of control and TSH-desensitized membranes with CT (100 μg/ml) in the presence of optimal concentrations of GTP (1 mM), Mg^{2+} (10 mM), and inorganic phosphate (300 mM), did not show any difference indicative of a TSH-induced change in the susceptibility of α_s subunits of G_s to be ADP ribosylated by CT (not shown). In contrast TSH treatment of slices reduced the ADP ribosylation of the 40 kDa PT substrate by 40% to 60% (Figs. 2–5).

In view of the differential effects that nucleotides have on the labeling of the 40 kDa substrate in thyroid membranes (18), we studied these variables, as well as the influence of membrane protein concentration and time of incubation on the labeling observed in control *vs.* TSH-desensitized membranes. In the absence of added nucleotides, very little ADP ribosylation of the 40 kDa was obtained. Because of this, all ADP-ribosylation studies except one (see below) were performed in the presence of saturating concentrations of nucleotides. The decrease in labeling of the 40 kDa band was independent of the amount of membranes assayed (Fig. 2), the time of incubation (Fig. 3), or the nucleotide used to support the ADP-ribosylation reaction.

Figure 4 presents a photograph of a long exposure

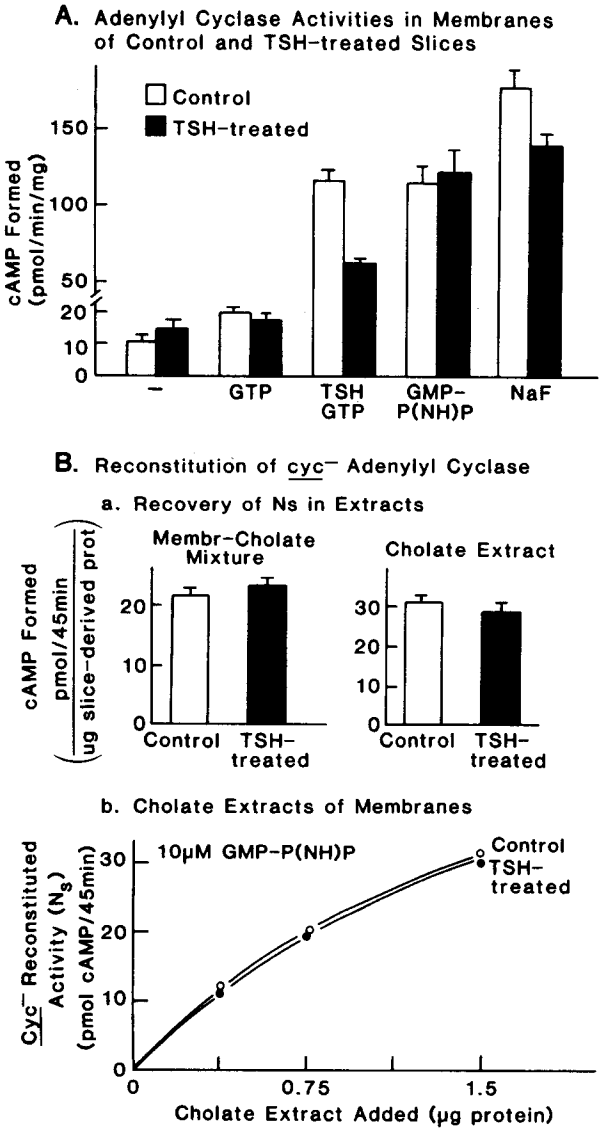

Fig. 1. Properties of Thyroid Adenylyl Cyclase Activity in Control and TSH-Desensitized Membranes

A, Guanine nucleotide-NaF-, and TSH-stimulated activities in membranes of control (□) and TSH-treated (■) thyroid slices. B, Reconstitution of *cyc*$^-$ S49 cell adenylyl cyclase by G_s of membranes from control (□ and *symbols*) and TSH-treated (■ and *symbols*) thyroid slices. Ba, Comparison of the efficiency of the extraction procedure. Membranes from control (0.80 mg protein) and TSH-treated (0.79 mg protein) slices were mixed with cholate and the mixtures either assayed before (*left section*) or after (*right section*) separation of the solubilized extract (0.42 mg protein from control and 0.40 mg protein from TSH-treated membranes) by the procedure described in *Materials and Methods*. Bb, Reconstituting activity in cholate extracts at varying concentrations of the extracted protein in the reconstitution assays. For details see *Materials and Methods* and Ref. 48.

autoradiogram of membranes ADP ribosylated with and without addition of GTP. Higher concentrations of GTP were necessary to enhance labeling of the 40 kDa band in membranes from TSH-treated slices than in control membranes. Thus, at low concentrations (0.01–0.03 μM), GTP had no effect on the labeling of the 40 kDa

[32P]ADP·ribosylation of Membranes from Control or TSH-Desensitized Thyroid Slices with Pertussis Toxin

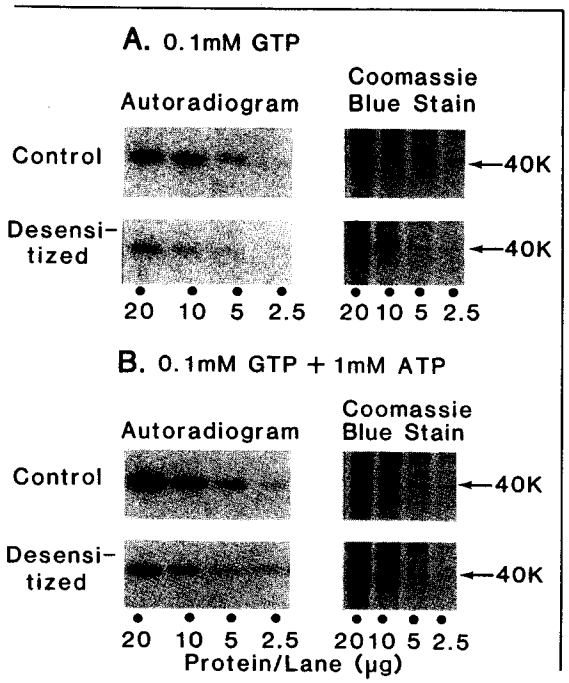

Fig. 2. Effect of TSH-Treatment on ADP Ribosylation of the 40 kDa Band in Thyroid Membranes Assayed at Varying Concentrations of Membrane Protein in the Presence of 0.1 mM GTP Alone or in Combination with 1.0 mM ATP

Membranes were diluted to the identical protein concentration and subjected to ADP ribosylation incubations lasting 30 min. *Left panels*; Autoradiograms; *right panels*; photographs of the Coomassie blue stained gels subjected to autoradiography.

[32P]ADP-ribosylation of Membranes from Control or TSH-Desensitized Thyroid Slices with Pertussis Toxin

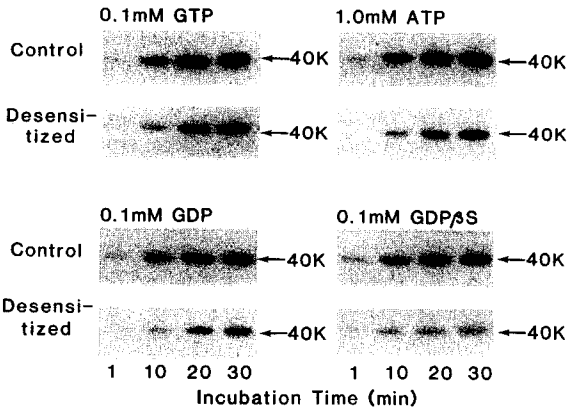

Fig. 3. Effect of TSH Treatment on ADP Ribosylation of the 40 kDa Band in Thyroid Membranes Assayed at Various Times of Incubation and in the Presence of the Indicated Nucleotides.

Membranes from control and TSH-treated slices were at 4 μg protein/assay.

[32P]ADP·ribosylation of Membranes from Treated Thyroid Slices with Pertussis Toxin

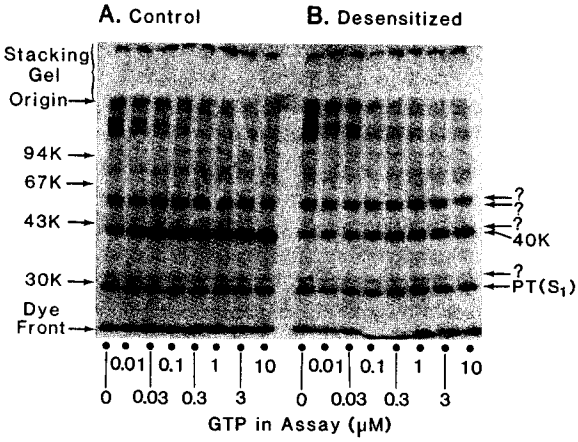

Fig. 4. Nucleotide-Independent ADP Ribosylation by PT of Components in Membranes from Control and TSH-Treated Slices and Effect of Low Concentrations of GTP on ADP Ribosylation of 40 kDa PT Substrate(s)

Incubations were for 30 min as described in *Materials and Methods* except that PT and membranes were 20 μg/ml and 18 μg/assay, respectively, and that 10×10^6 cpm [32P]NAD^+ were used. The GTP concentrations were varied as shown. Migration of molecular weight marker proteins (in thousands) are shown on the *left side* of the photograph and bands commented on in the text are indicated by *arrows* on the *right side* of the photograph of the autoradiogram. Autoradiography was for 4 days at −70 C.

band in T-desensitized membranes while as little as 0.01 μM GTP stimulated its labeling in controls. The decreased ADP ribosylation of the 40 kDa band by PT in membranes was dependent on the concentration of TSH and the time of incubation with the hormone (Fig. 5). The significant reduction in the labeling of the 40 kDa band in membranes from TSH-treated slices was not due to increased hydrolysis of GTP or ATP. Hydrolysis assays were done using [γ^{32}P]GTP or [γ^{32}P] ATP under conditions identical to those used in the ADP-ribosylation assays except for omission of [32P] NAD^+. Neither control nor desensitized membranes (<20 μg protein/assay) hydrolyzed significant amounts of the [32P]NAD^+ (not shown). When the membrane protein content was raised to 60 μg/assay, approximately 55% of the original [32P]NAD remained at the end of 30-min incubations but this was the same for control or TSH-desensitized membranes. Further, the ADP ribosylation of the 40 kDa band was also less when assays were done in homogenates from TSH-treated slices (not shown). The TSH effect was also not related to differences in loading of gel slab tracts (Fig. 2). A band of M_r *ca.* 41,000 was also radiolabeled in membranes from control and TSH-exposed slices. This band was unaffected by GTP and increments in the labeling of the 40 kDa band effectively obscured the labeling of the 41 kDa band. Further, additional GTP-independent labeling of bands approximately 54, 56, and 28 kDa was also observed. The intensity of their labeling was not affected by the initial incubation of

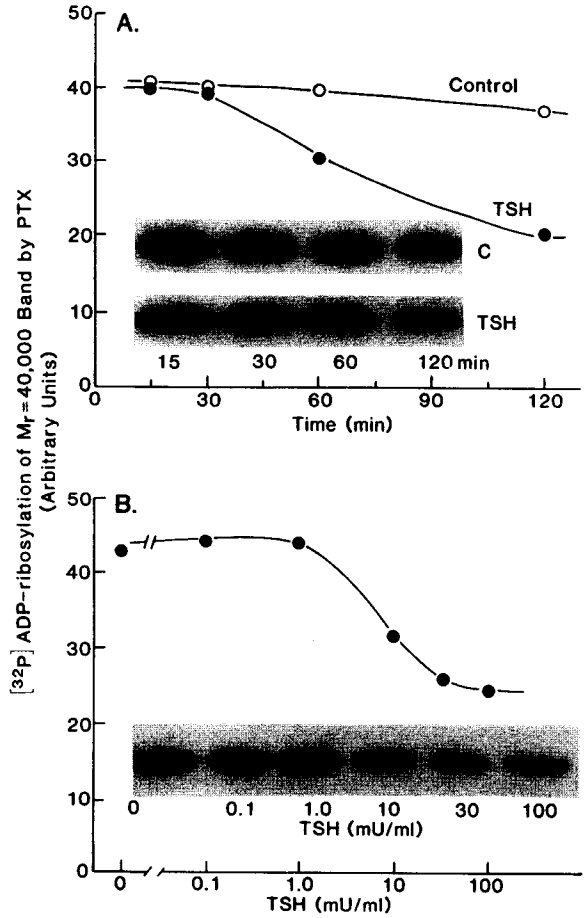

Fig. 5. Time and Concentration Dependence of TSH-Induced Decreased PT Catalyzed ADP Ribosylation of the 40 K Protein in Native Thyroid Membranes

A, Slices were incubated in Krebs-Ringer bicarbonate buffer with or without 100 mU/ml TSH. At the indicated times slices were washed, membranes were prepared and subjected to ADP ribosylation as described. The scale above the X axis is for the autoradiographies and below for the densitometric analysis. B, Slices were treated as in A but varying the concentration of TSH.

slices with TSH (Fig. 4). Thus, the decreased labeling of the 40 kDa PT substrate after TSH is unlikely to be a nonspecific effect. The ADP-ribosylated band at 28 kDa represents the auto-ADP-ribosylated S_1 subunit of PT.

Effects of Prostaglandin E_1 (PGE_1)

Previous studies demonstrated that an initial incubation of thyroid slices with PGE_1 induced desensitization to subsequent stimulation of cAMP accumulation to PGE_1 but not to TSH (1, 3). We investigated whether PGE_1 would also cause a change in the susceptibility of the 40 kDa substrate to ADP ribosylation by PT. Figure 6 shows the effect of pretreatment of thyroid slices with PGE_1 and TSH on basal (0.1 mM GTP), PGE_1-, and TSH-stimulated adenylyl cyclase activities in membranes prepared from these slices. The desensitization caused by PGE_1 is also of the homologous type. PGE_1 produced desensitization only to its own stimulation and in contrast to the results with TSH, ADP ribosylation of the 40 kDa band in PGE_1 desensitized membranes was unaltered (Fig. 7).

Effect of PT

The effect of PT pretreatment of thyroid slices on TSH-induced desensitization of the adenylyl cyclase was investigated with several PT concentrations (10, 50, and 1000 ng/ml) for 1, 3, and 6-h incubations. Membranes were assayed for adenylyl cyclase activity and for [^{32}P]ADP ribosylation of the 40 kDa band to test for presence of unaffected 40 kDa substrate. Although the incubation of thyroid slices with PT significantly reduced the subsequent ADP ribosylation of the 40 kDa band by the toxin, it did not completely prevent the incorporation of [^{32}P]ADP ribose into the 40 kDa band nor did it modify the TSH-induced refractoriness (not shown).

Unmasking of 40 kDa Substrate Affected by TSH Treatment with Lubrol PX

In view of our previous finding that addition of Lubrol PX to membranes before ADP ribosylation markedly enhances (10- to 40-fold) the incorporation of [^{32}P]ADP-ribose (27), we investigated whether the effect of TSH to decrease ADP ribosylation by PT would persist after such a treatment. Figure 8 shows treatment of membranes with increasing concentrations of Lubrol PX resulted in a progressive unmasking of the PT substrate. Although TSH-desensitized membranes showed decreased ability to accept ADP ribose at low detergent concentrations, under conditions leading to maximal labeling (*e.g.* high detergent), the 40 kDa PT substrate is equally ADP ribosylated in membranes from control and TSH-desensitized slices.

DISCUSSION

This work describes the first observation indicating a decreased susceptibility of one of the PT substrates to be ADP ribosylated by this toxin in membranes from tissues treated with a trophic hormone. Glucagon-induced desensitization of MDCK adenylyl cyclase, which is of the heterologous type (26), was associated with increased labeling of the PT substrate (*ca.* 40 kDa and referred to as G_i). Since the ADP ribosylation of the 40 kDa thyroid membrane protein by PT is quite dependent on guanine nucleotides it is highly possible that it is a G-type transducing protein (5). However currently available data do not permit unambiguous identification of the thyroid 40 kDa as G_i. In the thyroid, TSH stimulation of cAMP formation is attenuated by both α_2 adrenergic (15) and muscarinic cholinergic (16) receptor activation; both processes are thought to be transduced by G_i (28, 29). Although PT pretreatment prevented α_2, but not muscarinic, induced inhibition of cAMP (17) another report (25) found that PT did not alter the action of α_2 agonists, or of TSH, on cAMP metabolism. In fat (30)

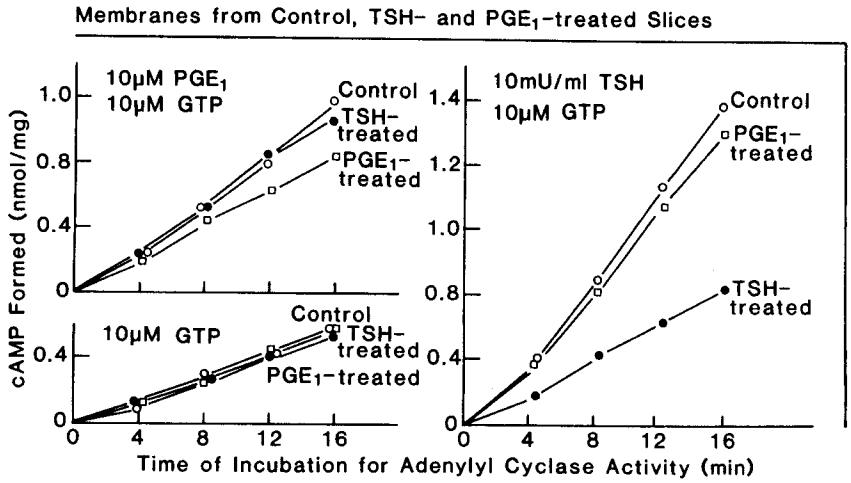

Fig. 6. Comparison of the Effect of Treating Thyroid Slices with TSH to that of Treating Slices with PGE_1 on Basal, TSH-, and PGE_1-Stimulated Membrane Adenylyl Cyclase Activity

Slices were incubated in Krebs-Ringer bicarbonate buffer containing 0.05% ethanol without (control) and with addition of 10 mU/ml TSH (TSH-treated) or 10 μM PGE_1 (PGE_1-treated). Membranes were prepared as described and adenylyl cyclase activities were measured in presence of 0.05% ethanol, 10 μM GTP without (*lower left*) and with addition of PGE_1 (*upper left*), or TSH (right).

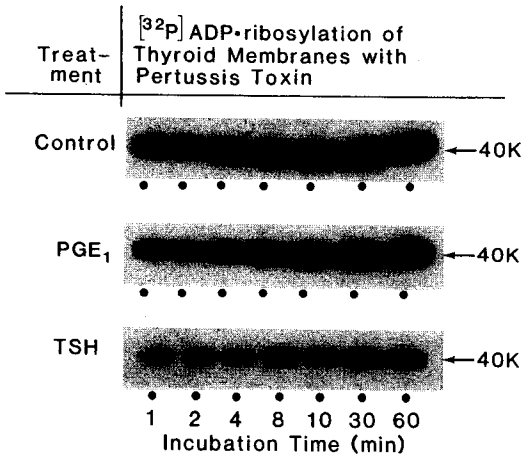

Fig. 7. Lack of Significant Effect of Treatment of Thyroid Slices with PGE_1 on ADP Ribosylation of 40 kDa Component(s) by PT

ADP ribosylation of membranes from control, PGE_1-, and TSH-treated slices was for the indicated times, using conditions described in *Materials and Methods*.

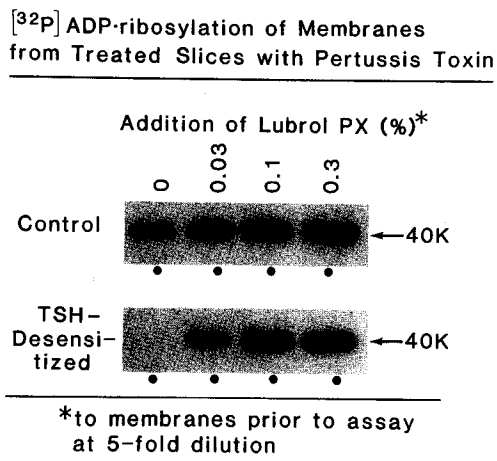

Fig. 8. Latency of PT Substrate Activity in Thyroid Membranes Prepared from Control and TSH-Treated Thyroid Slices

Control and TSH desensitized membranes (60 μg protein/ml) were pretreated for 30 min at 4 C with varying concentrations of Lubrol PX and then subjected, after a 5-fold dilution, to ADP ribosylation by PT for 30 min at 32 C. Autoradiography was for 4 h at −70 C.

and brain (31, 32) PT ADP ribosylates two proteins differing by as little as one or two kDa. The two PT substrates observed in brain, of 39 (called G_o) and 41 kDa (called G_i), respectively, have been purified. Only the 41 kDa PT substrate acts as an inhibitor of adenylyl cyclase (32) yet both proteins significantly increase agonist affinity for the reconstituted muscarinic receptor (34). There is immunochemical evidence of a 40 kDa PT substrate in both C6 glioma cells and in neutrophils that differs from G_i and G_o (35, 36). The 40 kDa band ADP-ribosylated in thyroid membranes by PT probably represents a mixture of substrates of which one is possibly G_i, plus one or more other substrates of as yet undefined function.

The decreased ADP ribosylation of the 40 kDa substrate by PT in native (no detergent added) TSH-desensitized membranes could reflect differential loss of a PT substrate from the desensitized membranes during their preparation. This is unlikely since the reduction of labeling was also seen in homogenates (not shown). Incubation of thyroid slices with TSH might alter the 40 kDa polypeptide so that it is not as good a substrate for PT. Thus, TSH which also activates protein kinase C (37) at doses similar to those in this study could phosphorylate the 40 kDa substrate and interfere with its ADP ribosylation by PT. Such phosphorylation by protein kinase C of the G_i of human platelet membranes has been reported (38). However, that report did not examine the effect of such phosphorylation on the ability of PT to ADP ribosylate its substrate. The decrease was also not due to an increased hydrolysis of

[^{32}P]NAD$^+$ or any of the other nucleotides required for optimal labeling. The effect was specific since labeling of other proteins was not decreased in membranes from slices incubated with TSH (Fig. 4). At this time we do not know whether one or all these putative different species of PT substrates is affected by TSH treatment nor what its physiological significance is.

The effect of TSH was not reproduced by PGE_1 which reproduces many actions of TSH on thyroid intermediary metabolism including desensitization of the adenylyl cyclase (4). However the extent of homologous desensitization caused by PGE_1 was significantly less than the TSH-induced refractoriness (Fig. 6). It is possible that PGE_1 desensitization in the thyroid affects a G protein that is distinct and of lesser abundance than that affected by TSH. The PGE_1-induced desensitization of adenylyl cyclase in thyroid tissue appears to be a process distinct from that of TSH (19). Hsia *et al.* (39) have recently concluded that [^{32}P]ADP ribosylation of the PT substrate in PGE_1-desensitized fibroblasts did not differ from controls. Thus, unlike TSH, and in spite of inducing a homologous type of desensitization, PGE_1 does not concomitantly affect the susceptibility of a PT substrate to be ADP ribosylated.

Since TSH was reported to increase endogenous ADP ribosyltransferases (21, 22) it is possible, that as a consequence of incubation of slices with the hormone, less substrate would be available for ADP ribosylation by PT. Kohn and collaborators (22, 23) have described an ADP-ribosyltransferase activity in bovine thyroid membranes with a substrate of M_r *ca.* 40,000 but such TSH sensitive activity was not found in FRTL-5 cells (24). We found little or no TSH-responsive endogenous ADP-ribosyltransferase activity in membranes. Although some labeling of 54 and 56 kDa bands was observed, it was not modified by TSH treatment nor was it sensitive to GTP (Fig. 4). Moreover, if endogenous ADP ribosylation of the 40 kDa were causally related to the observed decrease in PT-catalyzed ADP ribosylation of the 40 kDa substrate in TSH-desensitized membranes, it would not, as it was, be reversed by detergent pretreatment of the membranes (Fig. 8).

PT pretreatment did not reproduce or prevent the TSH induced desensitization of adenylyl cyclase in agreement with the recent results of Hirayu *et al.* (25). Thus, the decreased ADP ribosylation of a 40 kDa substrate by PT is likely to be a consequence of TSH action rather than the cause of desensitization. Involvement of the PT substrate in desensitization was suggested in some systems since PT treatment prevented refractoriness of adenylyl cyclase to glucagon in cultured hepatocytes (40) and to arginine vasopressin in cultured rabbit renal cortical collecting tubule cells (41). It is tempting to suggest therefore, that the effect of TSH observed here, may be quite specific for the mode of action of this hormone, as opposed to representing a general phenomenon accompanying desensitization events. This is supported by results obtained with PGE_1.

The biochemical reaction(s) involved in the changes that lead to TSH-mediated decreased ADP-ribosylation of the 40 kDa substrate are unknown. However, the results with exposure of membranes to Lubrol PX suggests that the TSH-induced change is one of compartmentalizing the 40 kDa substrate. Recently, Moss *et al.* (42) reported that ADP ribosylation of holotransducin by dithiothreitol- and ATP-activated PT is affected variably by detergents, from being slightly inhibited to maximally stimulated (at the most 3- to 4-fold) using cholate, phosphatidyl choline, and lysophosphadtidyl choline. If, however, membranes are pretreated with detergents (4 C, 30–60 min), the stimulation by detergent is 5–10 times greater than the values reported in Ref. 45. It is possible the Lubrol-sensitive compartment of the 40 kDa substrate is a physical compartment, such as a subclass of vesicles or a chemically distinct compartment such as may arise on phosphorylation of the 40 kDa substrate. Both these changes occur in β-adrenergic receptors during homologous desensitization (43). In the first case Lubrol PX would act by disrupting membrane integrity and increasing the physical availability of the substrate to PT. In the second case Lubrol PX would make the substrate more accessible to a phosphoprotein phosphatase, leading to a return of the substrate to its PT-sensitive form.

Another possibility relates to the subunit dissociation reaction that accompanies activation of G proteins, which are heterotrimers of composition $\alpha\beta\gamma$ (5–7). Rhodopsin, the photon receptor, can replace receptors that couple to purified PT substrates (44, 45). Photolyzed rhodopsin, but not the dark protein inhibited the ADP ribosylation of purified G_i by a mechanism that is thought to be due to dissociation of the $\beta\gamma$ complex from the heterotrimeric G_i and concomitant loss of the ability of the activated α-subunit to be ADP ribosylated by PT (46). Thus, TSH treatment might facilitate dissociation of the 40 kDa PT substrate and the dissociated complexes of the G protein could segregate into distinct membrane compartments before and/or during homogenization, creating vesicles with 40 kDa substrate but deficient in the $\beta\gamma$ complexes thought to be required for optimal ADP ribosylation (32, 46). In this case, Lubrol PX would enhance ADP ribosylation by promoting the reassociation to the trimeric complexes.

MATERIALS AND METHODS

Reagents

[^{32}P]NAD, [γ-^{32}P]GTP, [γ-^{32}P]ATP, and [α-^{32}P]ATP were synthesized, purified, and provided by the Molecular Endocrinology Core Laboratory of Baylor College of Medicine Diabetes and Endocrinology Research Center. PGE_1 was purchased from Sigma Chemical Co. (St. Louis, MO) and [^{3}H]cAMP from Amersham (Arlington Heights, IL). TSH (8.7 U/mg) was kindly provided by the Hormone Distribution Officer, National Institutes of Diabetes, Digestive and Kidney Diseases, NIH. PT and CT were obtained from List Laboratories (Campbell, CA). All other nucleotides and materials were as described in the preceding article (27).

Methods

Desensitization of Thyroid Slices Induction of desensitization of thyroid tissue was done by incubating 1–3 g bovine

thyroid slices previously washed with cold 250 mM sucrose in 10 mM Tris-HCl buffered saline (pH 7.4), at 37 C in Krebs-Ringer bicarbonate buffer (pH 7.4) containing 1 mg/ml BSA and 1 mg/ml glucose (5 ml/g wet tissue; 95% O_2-5% CO_2 gas phase) without and with 100 mU/ml TSH or 10 μM PGE_1. After 2 h of incubation the slices were washed several times with buffered saline plus sucrose at 22 C for 1 h. They were then minced with scissors, suspended in 20 mM Tris-HCl (pH 7.6), 1 mM EDTA, and 250 mM sucrose at 4 C (1 g/3 ml buffer) and homogenized in the cold for 15 sec using the lowest setting of a Polytron homogenizer. This homogenate was diluted (vol/vol) five times with the above buffer minus sucrose and further homogenized with 10 strokes in a Dounce homogenizer with a loosely fitting pestle. The homogenate was filtered through a no. 12 silk screen and centrifuged at 4 C at 800 × *g* for 10 min. The supernatant was used to obtain the membrane fractions as described in the preceding paper (27). These membranes were used for measurement of the activities described below.

Unless otherwise stated, adenylyl cyclase activities were determined in 0.3 mM [^{32}P]ATP (10 × 10^6 cpm), 1 mM [^{3}H]cAMP (10,000 cpm), 3 mM $MgCl_2$, 1 mM EDTA, 20 mM Tris-HCl, pH 7.6, 10–20 μg thyroid membrane protein and a nucleoside triphosphate regenerating system composed of 20 mM creatine phosphate, 0.2 mg/ml creatine phosphokinase and 0.02 mg/ml adenylate kinase (47). Incubations were in 50 μl at 32 C for 10 min and activity was linear with respect to time and amount of protein. For determination of activities of S49 *cyc*$^-$ membranes the $MgCl_2$ concentration was raised to 10 mM and incubations were for 45 min. Reactions were stopped and the [^{32}P]cAMP formed was isolated as described (48). Time course incubations were in 0.5–2.0 ml reaction mixtures which had been prewarmed at 32 C for 5 min. The assay was initiated by the addition of membranes, and at appropriate times 50-μl aliquots were withdrawn and added to 100 μl adenylyl cyclase stop solution (47).

Extraction of G_s Activity and Selective Inactivation of Coextracted Adenylyl Cyclase Activity Guidelines established by Dighe *et al.* (49) for assaying G_s in rat liver membranes were followed. Briefly, membranes (5–20 mg/ml) were mixed with extraction medium to give a final concentration of 1% cholate, 25 mM Na-HEPES (pH 7.8), 1 mM EDTA, and 10 mM $MgCl_2$. The membrane-cholate mixtures were kept on ice for 60 min. with occasional mixing, the EDTA concentration was increased to 10 mM, and mixtures were centrifuged at 4 C for 60 min. at 100,000 × *g*. The pellet was discarded and the supernatants were incubated at 30 C for 5 min to inactivate the coextracted C activity. This extract was used for reconstitution of the NaF- and GMP-adenyl-5′-yl imidophosphate-stimulated *cyc*$^-$ adenylyl cyclase activities in the presence of KCl and β-mercaptoethanol (49). To verify the *cyc*$^-$-reconstituting activity in thyroid membrane extracts relative to that of pure human erythrocyte G_s, parallel experiments were performed.

To measure *cyc*$^-$-reconstituting activity, thyroid membrane extracts (5 mg/ml) or purified G_s (0.08 mg/ml) were diluted 20- and 500-fold, respectively, in 10 mM Tris-HCl (pH 7.6), 1 mM EDTA, and 1% BSA, then, further diluted 2-fold in 40 mM 2-mercaptoethanol containing 0, 1, or 3 M KCl. Samples of 10 μl from the diluted extracts or purified G_s were mixed with an equal volume of *cyc*$^-$ membranes (20 μg) and kept at 4 C for 15 min. The incubations for determination of the reconstituted activities at final concentrations of KCl of 0, 100, and 300 mM, were then initiated by the addition of 30 μl reagents described above for the adenylyl cyclase assay. KCl has differential effects on the *cyc*$^-$-reconstituting activities of extracts or purified G_s. GMP-P(NH)P-stimulated activities were better seen at 300 and 100 mM KCl, while the NaF-stimulated activity was observed better without KCl (not shown). The significance of the inhibition of NaF-stimulated activity by high KCl concentrations is unknown. The KCl dependency of the GMP-P(NH)P-stimulated activity was proportional to the amount of extract. Cholate extracts could be stored at −70 C for up to 3 months without loss of activity. Studies in which reconstituting activities in control and desensitized membrane extracts were compared (see Fig. 1), used a single batch of *cyc*$^-$ membranes. Protein was determined according to Lowry *et al.* (50).

Radiolabeling of the PT substrate in 4–10 μg protein of control, TSH-, and PGE_1-desensitized membranes, electrophoresis on 10% polyacrylamide gel slabs, and autoradiography of stained and dried slabs were done as described previously (18). The toxin was activated at 32 C for 20 min with 25 mM DTT and 0.02% BSA, and the indicated concentrations of ATP, GTP, GDP, and GDPβS. The reactions were started by addition of [^{32}P]NAD (10 μM final). Experiments in which the reaction was started by the addition of thyroid membranes or activated toxin resulted in similar patterns of radiolabeling.

Assessment of hydrolysis of [^{32}P]NAD^+, [γ-^{32}P]ATP, and [γ-^{32}P]GTP by control and desensitized membranes (data not shown) was done under the same conditions of incubation as the ADP-ribosylation assays. The incubation media (2 μl containing 1.5 × 10^6 cpm appropriate nucleotide) together with UV-absorbing standards were spotted onto precoated polyethyleneimine cellulose plates and subjected to ascending TLC with either 1 M LiCl, 0.8 M ammonium sulfate (pH 5.7), or 2 M sodium formate (pH 4.0). Migration of the applied radioactive materials was determined by liquid scintillation counting.

All experiments were performed at least three times, and representative results of the replications are presented. ADP ribosylation of membrane fractions was done in duplicate and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. The results on adenylyl cyclase measurements are means ± SD of triplicate determinations.

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